Determination of Nitrogen and Protein Content of Meat and Meat Products

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Chemical and instrumental methods for determination of nitrogen and protein are reviewed for their mode of action and utility in analysis of meat proteins and products. Although the Kjeldahl digestion method is satisfactory for determining total nitrogen, it is imprecise for determining total protein content. Presence of variable amounts of nonprotein nitrogenous components and of connective tissue proteins such as collagen and elastin produces error if the formula (N × 6.25) is used to calculate crude protein. Such fibrous proteins have higher nitrogen levels (over 18%) than other muscle proteins (about 16%), and a higher than actual protein value will be determined unless a lower conversion factor is used to correct for their content. To determine meat protein content more accurately, a combination of Kjeldahl determination with one or more additional tests to correct for nonprotein and fibrous protein content is recommended. The choice of the additional method(s) is based on the user's requirement for protein characterization, available time, type of meat product, and sample size.

During the last decade, the U.S. Department of Agriculture has conducted research on meat and meat products to answer concerns of various consumer groups regarding the nutritional and health aspects of animal-based products. Specific concerns include the level and types of proteins and fats, as well as the value of the cure additives salt and nitrite. Consumers want an indication of the compositional levels in these products as purchased. With fresh meat products such as steaks, chops, and roasts, the amount of lean or fat tissues usually can be seen by the market shopper. With ground fresh meats and processed meats, however, variable amounts of fatty and connective tissues and nonmeat additives could be included without obvious changes in overall appearance. Consequently, legislation and analytical methods have been required to define and guarantee nutritional quality for such products.

Meat is defined in the Federal Register (1) as "any edible portion of the carcass of any cattle, sheep, swine, or goats, exclusive of lips, snouts, ears, caul fat, leaf fat, kidney fat, and other visceral fat, and exclusive of all organs, except the heart, tongue, and esophagus." Skeletal meat is "skeletal muscle tissue with accompanying fat that has been attached directly to bone, including that from the diaphragm and cheeks after they are trimmed to remove glandular tissue." Definitions for meat for the European market (2) are similar and

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include the animals listed above. Both sets of definitions for meat refer to the muscle tissue and associated connective tissues of domesticated animals and do not include the edible flesh from poultry, seafood, or game (1, 2).

Such definitions for meat are for source and location and do not contain compositional analysis. The gross composition for moisture, fat, and protein of various adipose and skeletal muscle tissues used in meat processing (Table 1) vary from lows of 1% protein and 8% water to 20% and 70%, respectively. In the most general sense, the combination of moisture and protein in meat constitutes the lean portion.

The wide variability in composition of the known components and the presence of uncharacterized substances in meats of unknown history prevent a strict characterization by defined content. The interrelationship of the compositional components in fresh meats with linear correlation coefficients generally greater than 0.95 (Table 1) allows use of predicted values for multi-analysis instrumental methods. For many skeletal tissues, moisture and protein vary in direct proportion, while fat content is inversely proportional to the other 2 components. Muscle proteins can be separated in fresh unheated meats on the basis of solubility in aqueous solutions of low or high ionic strength into 3 fractions—sarcoplasmic, myofibrillar, and stromal (3). When meat tissues are processed or heated, however, such fractionation methods are not usable because of heat denaturation and chemical interactions.

Table 1. Composition of typical meat processing materials[a,b]

Material	Protein, %	Fat, %	Moisture, %
Fat pork	0.9	91.7	8.1
Skinned jowls	7.5	71.2	20.9
Pork trimmings 50/50	9.1	54.5	35.9
Pork cheek meat	17.2	21.8	60.2
Beef chuck meat	18.3	11.4	69.4
Fresh bull meat	20.1	8.8	70.1

[a] Source of data: C. E. Swift, U.S. Dept of Agriculture, ERRC, Philadelphia, PA, personal communication, 1980.
[b] Linear correlation coefficents for these data: protein : fat, $r = -0.993$; protein : moisture, $r = 0.988$; fat : moisture, $r = -0.999$.

Analytical Methods for Nitrogen and Protein

Stewart (4) has indicated that the state of development of analytical methods in food products can be considered *adequate* for total nitrogen, *substantial* for most amino acids, and *conflicting* for total protein and some amino acids. Because proteins are an important part of human nutrition, various methods have been developed for their determination. These procedures make use of specific components of the proteins, i.e., peptide linkages, amino groups, selected amino acids, or attached carbohydrates, to quantitate protein content. Those methods that have been applied to determination of protein content of meat and meat products can be divided into 10 classes: (a) nitrogen content, (b) peptide linkages, (c) primary amino groups, (d) aromatic amino acids, (e) basic amino acids, (f) imino acids, (g) sulfur amino acids, (h) amides, (i) carbohydrates, and (j) "crude" dry protein. Some of these have been adopted at various times as AOAC official methods, but most have been developed or used only for specific purposes such as estimation of connective tissue proteins. Each of the 10 classes of analytical determinants will be examined for its applicability to meat studies. Depending on the type of protein and the source, widely divergent values for protein content can be obtained from the application of these analytical methods to the same sample.

(a) *Nitrogen content.*—Determination of total nitrogen and conversion to protein content by an appropriate factor is the basis for 4 AOAC official methods (5) for protein measurement in meat products. These methods require that the sample be a food and that nitrogen not be present in significant amounts as nitrate, or as azo or nitroso groups. Method **24.027** refers to the standard Kjeldahl method, **2.057**. In this procedure, the sample is digested at high temperatures with concentrated sulfuric acid, sodium sulfate, a metallic catalyst, and sometimes hydrogen peroxide to convert nitrogenous substances to ammonium salts. Addition of concentrated alkali to this digest converts the ammonium salts to free ammonia which is distilled, collected, and titrated by various procedures. Two other methods (**24.028–24.036** and **24.037**) use the basic Kjeldahl digestion with variations in the determination of ammonia nitrogen to allow automation and increased determinations per time period. The first of these methods uses a common sample digestion with provisions for both phosphorus and nitrogen determinations. Ammonia nitrogen is measured by reaction with hypochlorite and phenate ion in alkali to produce a quinonechloramine which reacts further with phenate to produce indophenol, which can be measured spectrophotometrically at 630 nm. The second method refers to **7.021–7.024** wherein the sample is digested by the Kjeldahl method and the ammonia produced is steam distilled and determined by an automated procedure. These 3 methods are defined for determination of *total nitrogen.* Percent nitrogen is calculated from milliequivalents ammonia per g sample by multiplying by the factor 1.4007. This value can be converted to crude protein content by a second factor: 6.25. A recent AOAC method and apparently the only specific listing for *crude protein* in meat is the block digestion method (**24.038–24.040**) which is not automated, but which allows a greater number of determinations to be conducted per unit of time. This method also uses the standard Kjeldahl digestion followed by steam distillation and titration, and conversion with the 2 factors listed previously. Whereas the Kjeldahl method is recognized as accurate and precise for nitrogen determinations (4), its application for protein content in certain products has been questioned because of the value of the conversion factor. AOAC method **14.067** (5) states that "Protein = nitrogen × 6.25 except for wheat and its products in which protein = N × 5.7. For all analyses, report %N and conversion factor used. Other traditional and customary factors are 5.18 for almonds, 5.46 for peanuts and brazil nuts, 5.30 for tree nuts and coconuts, and 6.38 for dairy products." In certain other products such as organ meats which contain large quantities of nucleic acids, a correction must be made for their contribution to total nitrogen. Deoxyribonucleic acids (DNA) contain 16% nitrogen, and a considerable error would be introduced in terms of protein content. The apparent value for protein would therefore be inflated by the additive nitrogen quantities from the protein and DNA content.

Other methods have been developed to determine nitrogen content by a variation of the Dumas method (5) (**6.016–7.020**), which has been applied for analysis of animal feeds. In these methods, the sample is combusted and free nitrogen is determined. With the Dumas method, gas volume is determined, corrected to standard conditions, and converted to mass equivalent. More recent instrumental methods have utilized conversion of nitrogen to an activated form and determination by chemiluminescence (6) and measurement by a flow injection technique (7). These procedures show

high correlation with values determined by the Kjeldahl method because all measure nitrogen present in the sample.

(b) *Peptide linkages.*—Because proteins are linear polymers of amino acids linked together mainly by peptide bonds plus some other chemical bonds, measurement of peptide linkages provides an indication of protein content. The principal methods are ultraviolet or near infrared absorption and complexation with copper ions. In the AOAC biuret method, **7.010**, for animal feed (5), a stabilized alkaline copper reagent is used to form a complex with 2 or more peptide bonds. The color of the copper complex as determined spectrophotometrically indicates the content of protein peptide linkages. The biuret test is commonly used in biochemical measurements for soluble proteins, but the test is not very sensitive, and glycerol, ammonium salts, sugars, and Tris buffer interfere in the analysis. Insolubility of meat samples precludes use of the biuret method. A pulsed nuclear magnetic resonance system (8) has been proposed for measuring the complexed copper in insoluble matrixes. Absorption of ultraviolet light by the peptide amide bonds has also been used in biochemical measurements particularly in liquid chromatographic applications, specifically by measuring absorption at 205, 220, and 235 nm. These wavelengths, however, are susceptible to interference from light scattering and by absorption by nonprotein components. Absorption by the amide linkages in the near infrared region apparently requires that the particles be of identical size for reflectance techniques (9).

(c) *Primary amino groups.*—Because proteins contain primary amino groups, their determination has also been used for protein determinations. In the tenth edition of *Official Methods of Analysis* (10), 2 methods were listed as official first action under Meat Products, but they are not included in the present edition. Method **23.017–23.018**, the Van Slyke nitrogen method, measured the nitrogen gas released by reaction of nitrous acid with primary amino groups; **23.019**, the Sorensen formol titration method, measured the ionization of primary amino groups by titration in the absence and presence of formaldehyde, an amino complexation agent. The principal procedure for determination of primary amino groups is the standard amino acid analysis which determines the content after hydrolysis by reaction with ninhydrin to produce a chromogenic substance proportional to amino acid content (11). Alternative reactants for quantitation of primary amino groups include fluorodinitrobenzene, fluorescamine, and phenylisothiocyanate (12). A method proposed by Horstmann in 1978 (13) uses the reaction of ninhydrin with the unseparated hydrolysate for estimation of total amino acids and therefore total protein. In this method, the relative content of amino acids in each protein must be determined first, because each protein has a different weight conversion factor and the contribution of imino acids must be known and a correction made. Although the factors for proteins range from 0.083 for elastin to 0.147 for protamines, most proteins apparently have factors between 0.11 and 0.12. A similar method for meat hydrolysates using trinitrobenzene sulfonic acid was proposed by Arneth in 1983 (14) for use in an automated system along with determination of the specific amino acid hydroxyproline.

Although all proteins contain nitrogen, peptide linkages, and primary groups, they differ in their content of specific amino acids. Chemical reactivities of various individual amino acid side groups can be determined on intact proteins or following acid, base, or enzymatic hydrolyses. Conversion of the content of specific amino acids to total protein assumes that the content of that amino acid is constant, and that no alterations occurred during preparation. With the commonly used acid hydrolysis procedure (11), for example, destruction of tryptophan, sulfur amino acids, and amides may occur.

(d) *Aromatic amino acids.*—The aromatic amino acids are phenylalanine, tyrosine, and tryptophan. The more common methods measure the phenolic ring of tyrosine; these include measuring absorption at 280 nm, and use of Folin's reagent, the Lowry procedure, Millon's reagent, and the xanthoproteic test (12). Ultraviolet absorption at 280 nm also measures contributions from phenylalanine, tyrosine, and tryptophan as well as nucleic acid components. The Lowry method is widely used in biochemical analyses for low levels of soluble proteins. It requires only about 5–10 μg/mL, but Tris buffer, EDTA, thiol reagents, sucrose, oxidized lipids, sulfhydryls, purines, hexosamines, amine buffers, phenol, magnesium, and potassium interfere in the analysis. The mechanism is not completely known but appears in part to involve complexation of copper ions in alkaline solution with peptide linkages (biuret reaction), followed by univalent reduction of the copper, and reaction of this copper with the Folin–Ciocalteau reagent to produce the blue complex. Aromatic amino acids may also be involved in the oxidation-reduction reactions, because proteins low in aromatics often have lower apparent protein values. A recent commercial protein determination uses a procedure similar to that of Lowry, except bicinchoninic acid replaces the Folin reagent and interference from the above substances is eliminated (15). Millon's reagent is used in method **7.011**, and the xanthoproteic test is method **7.014** (5). The Hopkins–Cole method and the Adamkiewicz variation for tryptophan determinations are methods **7.012** and **7.013**, respectively (5). These 4 tests are AOAC surplus methods.

(e) *Basic amino acids.*—Basic amino acids in acid medium are positively charged and can bind certain dyes, providing an indication of protein content. The proteins in meat have differing binding parameters. Seperich and Price (16) found that the sarcoplasmic proteins bind 27.0 ± 2.1 ng Acid Orange 12 dye/mg protein, the myofibrillar proteins 36.0 ± 2.8 ng, and the stromal proteins including collagen only 14.4 ± 2.0 ng. Histones, because of their high content of basic amino acids, would be expected to bind much higher amounts but these would only present a problem with organ meats. Consequently, dye binding procedures for meat protein estimation yield inaccurately low values with meats that have a high content of connective tissues or collagen. Other procedures for basic amino acids involve titration curves, the Sakaguchi method for arginine, and the trinitrobenzenesulfonic acid method for the epsilon amino group of lysine (17).

(f) *Imino acids.*—The imino acids, proline and hydroxyproline, are important in the connective tissue proteins collagen and elastin. Proline is a component of most proteins, but is present in high levels in collagen (about 11–14%) along with about 35% glycine and 11% alanine (12, 18). Hydroxyproline is also present in collagen in high levels (11–14%) and in low levels in some plant cell walls (19) but is not present in the skeletal muscle proteins. Consequently, determination of hydroxyproline content has been used as an indication of the connective tissue content (principally collagens). In the Woessner method or its variations, protein is hydrolyzed completely or partially to free amino acids. The hydroxyproline residues are oxidized with Chloramine T or periodic acid and are coupled with dimethylaminobenzaldehyde (Erlich's reagent) to give a chromogen that is measured spectrophotometrically (20). The content of hydroxy-

proline is multiplied by a factor to calculate apparent connective tissue. If a 12% hydroxyproline level is assumed, the factor is 8 (21); if the level is assumed to be 14%, the factor is 7.14. Determination of the connective tissue content of meats has been studied in Europe where methods are being considered for analytical determination of the fat-free, connective tissue-free meat. In an interlaboratory study reported by Dransfield et al. (22), determination of hydroxyproline content was the least precise procedure examined, with values ranging from 0.7 to 1.5% connective tissue content on the same sample and with 95% of replicates differing by up to 0.2%, well above the 0.06% suggested value. The calculated mean determinations of nonfat, nonconnective tissue lean meat from the various laboratories ranged 1.3%, from 19.9 to 21.2%. Correlation for glycine : hydroxyproline in collagen is 0.918, and for proline : hydroxyproline 0.80. Isatin and Sirius Red are histochemical dyes that react with proline and have been used for quantitation of connective tissues (23).

(g) *Sulfur amino acids.*—The sulfur amino acids are cysteine, cystine, methionine, cystathionine, and taurine. Only the first 3 are found in most meat proteins. The principal method involves use of Ellman's reagent which reacts quantitatively by a disulfide exchange reaction with the free sulfhydryl of cysteine (24). Some cysteine is present in nonproteins such as glutathione, and the reagent is also subject to interference by thionucleophiles (25). Processing often oxidizes some of the free sulfhydryls, altering their apparent content. The level of sulfhydryls may also be measured by the nitroprusside reaction or by titration with iodoacetate.

(h) *Amide content.*—Alkaline distillation has been used to determine the amide content of proteins (26), although near infrared spectroscopy measures some of the amide bonds along with the peptide linkages. Release of ammonia from the amide linkage during alkaline hydrolysis is rapid. Asparagine is a somewhat stronger electrolyte than is glutamine, more susceptible to nucleophilic substitution, and more likely to lose an amide proton. Extended time of distillation can cause release of ammonia from arginine (about 2% of total content) and from rearrangement reactions of serine or threonine. The amide content of proteins ranges from 2% for silk fibroin and 4% for collagens to about 26% for wheat gliadin, with most proteins about 8–9% (26). The alkaline distillation procedure has been proposed as a rapid method (less than 30 min) for estimation of meat proteins (27, 28).

(i) *Carbohydrate content.*—Many proteins are actually glycoproteins and contain carbohydrate residues. Most membranes contain gangliosides, complexes of carbohydrates attached to lipids, often combined with proteins. The quantitation of these residues as a function of protein level has not been used to any great extent except for soy proteins (29). With this method, the sample protein is hydrolyzed with dilute acid, the hydrolysate is neutralized, and the sugar content is determined by specific enzymes. Galactose is present in collagens, milk protein, and soy protein, whereas mannose is the principal carbohydrate in gliadin, a wheat protein.

(j) *"Crude" dry protein.*—Crude protein content is often approximated indirectly by calculating the weight remaining after removal of moisture and fat. This can be accomplished by rendering with heat or microwave energy or by extraction with specific solvents, followed by drying. With fresh meats, the correlation with protein content is high because of the lean-to-fat compositional relationship, but correlation is poor with processed meats where additives such as salt contribute to the total weight, giving false results.

Nonprotein Nitrogenous Compounds in Meat Products

Muscle proteins contain various nonprotein components whose content has been proposed as an indicator of muscle protein content. Creatine and creatinine are both present in fresh striated muscle, particularly the white fibers, and can be readily measured. Dvorak (21) reported that creatine values were not linear for total proteins (N × 6.25) but were linear for net protein values (total protein − hydroxyproline content × 8). Normal creatine levels are 23 mg/g net muscle protein, and creatinine levels are 1 mg/g. The nitrogen from these 2 substances would give an apparent 48 mg protein/g net muscle protein. During storage, creatine slowly dehydrates to form creatinine (30).

The methyl histidines also have been proposed for meat protein estimations (31). Methyl histidines are present in both the actin and myosin myofibrillar proteins and in the sarcoplasmic dipeptide buffers anserine and balenine. These beta-alanyl histidines can be extracted readily in fresh meats and determined by reaction with *o*-phthalaldehyde. Their levels show species differences. If the protein is hydrolyzed, the methyl histidines can be separated on ion exchange columns. The nonfat, nonconnective tissue free protein contains 6 mg 3-methyl histidine/g protein nitrogen.

Nonprotein extracts have been prepared with specific denaturants such as trichloroacetic acid, phosphotungstic acid, or barium hydroxide–sulfuric acid. Studies have indicated that not all the proteins are removed by such treatments. With 5% trichloroacetic acid, 21% of the actomyosin and 4% of the hemoglobin remained in solution (32). Phosphotungstic acid and barium hydroxide procedures appeared to be more effective but disposal was considered hazardous. In this safety aspect, it has been proposed that pure potassium sulfate plus hydrogen peroxide in the Kjeldahl procedure provides the same results at high digestion temperatures as does the potassium sulfate-heavy metal catalyst (33). Elimination of this use of mercuric oxide, copper, or selenium is beneficial in regard to exposure of the analyst and disposal to the environment.

Nitrogen-Protein Conversion Factors

The Kjeldahl method incorporates the assumption that all meat proteins have a mean nitrogen content of 16% with the mixture of amino acids. Calculation of the nitrogen percentage of the amino acid residues indicates that 13 have percentages less than 16 (Tyr 8.6; Phe 9.5; Met 10.7; Glu 10.9; Leu, Ile, and Hypro 12.4; Asp 12.7; Cys 13.6; Thr 13.9; Val 14.1; Pro 14.4; Try 15.0). Nine amino acid residues have nitrogen percentages greater than 16 (Ser 16.1; Hylys 19.4; Ala 19.7; Lys and Gln 21.9; Gly 24.5; Asn 24.6; His 30.6; Arg 35.9). Ammonia has a nitrogen content of 58.3%. The aromatic amino acids (phenylalanine, tyrosine, and tryptophan), the sulfur amino acids (cysteine and methionine), the acidic amino acids (glutamic and aspartic), and the imino acids (proline and hydroxyproline) all have nitrogen content below 16%, whereas the basic amino acids (lysine, hydroxylysine, histidine, and arginine), the simple aliphatics (glycine and alanine), and the amides (glutamine and asparagine) have higher contents. Free ammonia may be present from bacterial deaminations or from breakdown of amide linkages.

From published amino acid analyses (26, 34, 35), the weight percent content of the aromatic, sulfur-containing, basic, and amide amino acids in various meat components and products can be calculated as well as the nitrogen conversion factor (Table 2). Accurate amino acid determinations for certain proteins are difficult to locate in the literature, particularly

Table 2. Content of amino acid types and nitrogen-protein conversion factor calculated from amino acid data for muscle proteins and meat products[a,b]

Sample	Aromatic	Sulfur	Basic	Amide	Conv. factor
Collagen	2.2–3.6	0.5–1.1	12.8–14.9	0.2–2.5	5.24–5.48
Myosin	6.1–7.6	4.2–5.7	13.2–19.2	4.2–4.6	6.0–6.4
Actin	11.3	5.1	15.1	3.4	6.20
Albumin	12.7	7.6	22.0	3.1	6.28
Myoglobin	7.3	1.7	25.9	1.8	5.76
Lean meat	10.2	3.5	19.3	4.9	5.92
Mixed tissue	9.1	2.7	17.6	4.6	5.76
Adipose tissue	7.7	1.8	15.2	7.1	5.75
Connective tissue	8.0	2.6	14.9	5.6	5.66
Frankfurter	7.7	3.4	16.6	5.7	5.80

[a] Refs 26 and 34 were used for calculation of collagen, myosin, actin, albumin, and myoglobin; ref. 35 was used for collagen and the meat products.
[b] Values are expressed in sum of the relevant amino acids (g/100 g).

those with separate amide and tryptophan determinations. With muscle components the range of conversion factors is wide; for actin and myosin the factor is close to 6.25, whereas for collagen it is much lower. With meat products, the calculated factors were all below 6.0. A recent report by Yamaguchi (36) indicated that 5.73 was a better value for meat protein conversion, with factors for fruits, vegetables, and soybeans even lower.

With meat proteins, this lowered value arises from the presence of variable amounts of endogenous and exogenous nonprotein nitrogenous components and of connective tissue proteins, i.e., collagen and elastin. The correct factor for conversion appears to be directly proportional to a separate quantitation of these nitrogenous components and collagen. This would apply particularly to processed meats, because there are no minimum protein standards for fresh meats. Processed meats, however, can contain various regulated additives (1), some of which (soy protein, gelatin, and milk protein) do contain nitrogen. Certain products, e.g., head cheese or chicken roll, contain added skin which is high in collagen.

Determination of collagen content by determination of hydroxyproline levels and conversion can be done to estimate connective tissue content. In these cases the correct factor must be used. If the factor for collagen nitrogen were 5.7 rather than 6.25, there would be an 8.8% difference in calculated protein. If the factor is actually 5.4, the difference is 13.6%. This difference then directly affects the accuracy in determination of protein in certain meat products. Recent regulations (1) on defining the minimum protein fat-free (PFF) values for certain processed pork products are intended to indicate the apparent amount of water added during processing. PFF values are calculated by dividing the percentage protein determined using a total nitrogen method by a value of (100 minus the fat percentage). A cooked ham with a calculated crude protein content of 18% and a fat content of 10% would have a PFF value of 18/90 or 20.0. This would fall below the required PFF standard of 20.5 for common and usual hams. The regulations provide for subtraction of nonmeat proteins and gelatin that were added during processing, which necessitates additional analyses. Ham that contains excessive levels of connective tissue will indicate unrealistically high crude protein content.

Recent studies in our laboratory have indicated that the ratio of amide nitrogen (determined by direct alkaline distillation under defined conditions) to total nitrogen (determined by Kjeldahl) reflects the collagen content of the meat, because collagens have a low amide content. Other experiments on processed meats have indicated that an extraction with hot distilled water and characterization of the extracts for nitrogen can aid in detection of excessive additives and detection of nitrogenous constituents of lesser nutritional value than the essential amino acids.

For most accurate meat protein analyses, a combination of Kjeldahl with one or more parallel determinations is recommended. With fresh meats, separate determination of collagen content is recommended to correct the nitrogen-protein conversion factor. If the factor 5.40 for pure collagen is used, then the correct factor for collagenous meats is $(6.25 - 0.0085A)$, where A is percent collagen in the total protein. If the hydroxyproline-collagen conversion factor of 8 is used, the correct nitrogen-protein factor is $(6.25 - 0.068B)$, where B is percent hydroxyproline. By this method, a meat product containing 50% collagen would use the nitrogen-protein factor 5.825 rather than 6.25. This 6.8% difference could affect the values determined for PFF calculations. The choice of the additional method(s) is based on the user's requirement for protein characterization, available time, type of meat product, and sample size.

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